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### Citation for published version:

McGorum, BC, Pirie, RS, Shaw, D, MacIntyre, N & Cox, A 2016, 'Neuronal chromatolysis in the subgemmal plexus of gustatory papillae in horses with grass sickness', *Equine Veterinary Journal*, vol. 48, no. 6, pp. 773-778. <https://doi.org/10.1111/evj.12530>

### Digital Object Identifier (DOI):

[10.1111/evj.12530](https://doi.org/10.1111/evj.12530)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Equine Veterinary Journal

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Received Date : 30-Jun-2015  
Revised Date : 06-Oct-2015  
Accepted Date : 14-Oct-2015  
Article type : Article

## **Neuronal chromatolysis in the subgemmal plexus of gustatory papillae in horses with grass sickness**

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**Keywords:** horse; grass sickness; subgemmal plexus; gustatory papillae; chromatolysis

### **Summary**

**Reasons for performing study:** Diagnosis of equine grass sickness (EGS) can be challenging. We hypothesised that subgemmal plexus neurons are chromatolytic in EGS. If correct, histopathologic examination of gustatory papillae biopsies could aid pre-mortem diagnosis of EGS, and EGS could represent a spontaneous model of subgemmal neuronal chromatolysis to facilitate study of the pathology of structures involved in taste.

**Objective:** To compare subgemmal plexi and gustatory papillae in EGS and control horses.

**Study design:** Observational study.

**Methods:** Conventional histology and immunohistochemistry were used to compare subgemmal plexi and gustatory papillae in post mortem samples from 10 EGS and 13 control horses.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/evj.12530

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**Results:** Chromatolytic neurons were present in all 57 EGS sections which had identifiable neurons, and in only one of 57 control sections. Blinded examination of all haematoxylin-eosin stained sections from each horse for chromatolysis facilitated accurate differentiation of EGS and control horses, with a sensitivity of 100% (95% CI 93.7-100) and specificity of 98.2% (90.6-100) for diagnosing EGS, however the presence of chromatolytic neurons in one control section indicated that multiple sections per horse must be analysed to achieve diagnostic accuracy. EGS was not associated with alterations in taste bud density or morphology, proportion of taste buds with neurofilament immunopositive intragemmal axons and proportion of taste buds containing cells undergoing apoptosis, suggesting taste buds had adequate neurotrophic support at the time of sampling. EGS horses had no detectable alteration in lingual gland morphology, but had increased proportions of apoptotic lingual serous gland cells.

**Conclusions:** While identification of chromatolytic subgemmal neurons in post mortem samples correctly differentiated EGS and control horses, further study is required to evaluate this technique for pre-mortem EGS diagnosis. EGS represents a spontaneous model of subgemmal neuronal chromatolysis that facilitates study of the pathology of structures involved in taste.

## **Introduction**

Diagnosis of equine grass sickness (EGS), a predominantly fatal, idiopathic acute multiple-system neuropathy, can be challenging [1]. EGS results in chromatolysis and/or degeneration of enteric neurons, peripheral autonomic neurons, lower motor neurons of the general visceral efferent nucleus of CN III and X and the general somatic efferent nuclei of CN III, V, VII and XII, and lower motor neurons in the spinal cord [2-5]. The predominant clinical signs of EGS

are attributable to intractable gastrointestinal ileus caused by severe enteric neuropathy [5]. We hypothesised that subgemmal plexus neurons are chromatolytic in EGS, since this plexus represents a specialised cephalic extension of the enteric nervous system [6]. While the equine subgemmal plexus has not been previously studied, in other species it controls processes ancillary to taste, including regulation of lingual glands and vasomotor control [7,8]. Subgemmal plexi underlie equine lingual gustatory papillae, which have been studied at gross, histological and scanning electron microscopy levels [9-12]. Two large vallate papillae located on the caudodorsal aspect of the tongue (Fig 1) have wide circular papillary clefts around central bulbous projections, into which open lingual glands (minor salivary glands). Foliate papillae, located on the caudolateral tongue, rostral to the palatoglossal arches, form numerous leaves separated by deep clefts (Fig 1). Taste buds, located in the epithelium lining papillary clefts, comprise groups of taste bud cells which are innervated by intragemmal nerve fibres.

As a preliminary step in determining the diagnostic utility of gustatory papillae biopsies in EGS diagnosis, we compared the morphology of subgemmal plexus neurons in haematoxylin-eosin stained sections of papillae collected post mortem from EGS and control horses. Further, the utility of EGS as a spontaneous model of subgemmal neuronal chromatolysis to facilitate study of the pathology of structures involved in taste was determined by assessing the effect of EGS on taste buds and lingual glands. Numbers and morphology of taste buds, proportions of taste buds containing intragemmal nerve fibres, proportions of taste buds containing apoptotic cells, proportions of apoptotic lingual gland cells and morphology of lingual glands were compared in EGS and control horses.

## Materials and methods

### Sampling

Samples of tongue, approximately 1 cm deep, including the entire cross section of vallate papillae or the entire length of foliate papillae, were collected from 13 EGS (median age 7 years, range 2-20) and 10 control (7.5 years, 3-30) mixed-breed and mixed-gender horses within 60 min of euthanasia. Vallate and foliate papillae were collected from 8 EGS and 7 control horses, only foliate papillae from 3 EGS and 3 control horses, and only vallate papillae from 2 EGS horses. EGS horses comprised 8 acute (6.5 years, 2-15) and 5 sub-acute (7 years, 3-20) cases, as previously categorised [13]. None of the EGS horses had chronic EGS. Acute and sub-acute EGS has an acute clinical course, with study horses being euthanased at a median of 24 h (range 6 h-5 days) after onset of clinical signs. EGS was confirmed by necropsy including histopathological examination of the cranial cervical ganglion [14]. Control horses were euthanased for reasons other than neurological disease, including laminitis (n = 4), lameness (2), undiagnosed chronic weight loss (1), hepatic abscess (1), peritonitis (1) and myopathy (1).

As a preliminary step to determining whether diagnostically useful biopsies could be collected from live horses, we also collected post mortem 'simulated biopsies' from the foliate papillae of 2 of these horses (one EGS, one control) using uterine biopsy forceps and placement of an oral gag.

### Tissue processing

Tissues were fixed in 10% formalin for at least one week before processing. Between one and 7 blocks were prepared from each papilla, yielding 24 blocks of vallate papillae and 41 blocks of foliate papillae from EGS horses and 20 blocks of vallate papillae and 40 blocks of foliate papillae from controls. Blocks were embedded in paraffin and 5 µm sections cut.

## Histology and immunohistochemistry

Histological assessment of subgemmal plexi and gustatory papillae was done on haematoxylin and eosin (H&E) stained sections from all blocks. Immunohistochemistry was used to detect pan-neuronal marker protein gene product (PGP9.5) [15], and neurofilament 2F11 to label lingual intrinsic fibres [8]. Representative single sections of foliate and vallate papillae from each horse were labelled using the following primary antibodies; rabbit polyclonal anti-PGP 9.5 (7863-0504)<sup>a</sup>, and murine monoclonal anti neurofilament 2F11 (M0762)<sup>b</sup> (see Supplementary Item 1 for detailed methodology). Cells undergoing apoptosis were labelled using a commercial kit (Apoptosis ApopTag Peroxidase In Situ Apoptosis Detection Kit)<sup>c</sup> which utilises TUNEL assay based technology to detect 3'-OH ends of double- or single-stranded DNA associated with apoptosis, according to the manufacturer's recommendations; antigen retrieval techniques were not used.

## Evaluation of sections

Number and morphology of all neurons in all (n = 125) H&E stained sections were scored by a single pathologist (A.C.) blinded to whether sections were from EGS or control horses. Neurons were identified by standardised scanning of entire sections at high magnification (200x or 400x), sections being considered to have chromatolytic neurons if at least one neuron had characteristic features of chromatolysis [4].

Average numbers of taste buds per papillary cleft were determined for all H&E stained sections, counting only taste buds with well-defined outlines as previously described [16,17]. Taste bud morphology was assessed for characteristic morphologic degenerative changes induced by experimental neurectomy [18]. The % of taste buds with intragemmal neurofilament immunopositive axons was determined on all sections. The % of taste buds containing at least one apoptotic taste bud cells was determined on single ApopTag labelled

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sections from foliate and vallate papillae from each horse. The % of apoptotic lingual mucous and serous gland cells were determined by examining 500 cells on single ApopTag labelled sections of vallate papillae from each horse.

#### Data analysis

As there were insufficient numbers of acute and sub-acute EGS horses, data for all EGS cases were pooled for analysis. Intergroup comparisons of age were made using a standard two-sample t-test. Analysis of differences in (i) numbers of perikarya, (ii) numbers of taste buds and (iii) numbers of taste buds per cleft per slide between control and EGS was done using generalised linear mixed-effect models (*glme*) with Poisson errors to account for the integer nature of data, with the horse from which samples were taken entered as the random effect to account for the repeated measures per horse [19]. For analysis of differences in percentage of taste buds which had neurofilament positive fibres, *glme* with binomial errors were employed. For analysis of percentage of total taste buds per horse that were apoptotic in either foliate or vallate sections, standard general linear models (*glm*) with binomial errors were done. Finally, differences between EGS and control horses in number of apoptotic lingual mucous and serous gland cells per 500 lingual mucous and serous gland cells, respectively, were analysed using *glm* with negative binomial errors. Analyses were done in R<sup>d</sup> (v3.1.1 © 2014 The R Foundation for Statistical Computing) with *glme* analyses using the *lme4* package (v1.1-7), and least square means and 95% confidence intervals (C.I.) for each group calculated using the *lsmeans* package (v2.11). Statistical significance was set at  $P < 0.05$ .

## Results

There was no statistically significant inter-group age difference (EGS  $\bar{x}$  =10.3 years; 95% C.I. 4.1-11.6, control 7.85; 6.0-14.6,  $P = 0.380$ ).

### General structure of the equine subgemmal plexus and gustatory papillae

There was no apparent difference in gross appearance of gustatory papillae from EGS and control horses. A complex network of neurons and nerve fibres resembling that of other species [6,7] was identified underlying equine gustatory papillae. Main ganglia (termed VP/VEG or Remak's ganglion when associated with vallate papillae) were located at the base of each papilla (Fig 2). These typically comprised several solitary neurons or small clusters of neurons located along a line projecting between the bases of papillary clefts. Deeper ganglia typically comprised groups of several neurons (Fig 2). Single neurons and ganglia were also located deeper within the tongue, particularly adjacent to lingual glands and between muscle bundles. The centre of many papillae contained several isolated neurons. Nerve fibres were evident emanating from, and surrounding, neuronal perikarya. A complex network of nerve fibres and axonal trunks was evident throughout the tissue, particularly within papillae, where they projected towards the gustatory epithelium, to innervate cells within taste buds which were located at terminal ends of papillary clefts, and occasionally to course through the squamous epithelium towards the tips of papillae. The apparently considerable inter-horse difference in density and complexity of the network of nerve fibres and axon trunks precluded meaningful objective comparison of the density of nerve fibres and axon trunks between EGS and control horses. Lingual glands were located deep to lingual papillae (Fig 2). Glands underlying vallate papillae (termed von Ebner's gland) typically comprised a single superficial mucous gland located in the centre of the base of the papilla, and numerous larger serous glands located near papillary clefts and interspersed throughout deeper muscle



bundles (Figs 2a, 5). Variations included absence of central mucous glands, mixed sero-mucous glands and mucous glands containing serous demilunes. Many serous, mucous and mixed seromucous glands were located beneath foliate papillae (Fig 2b).

Neuronal perikarya were identified in most sections from all papillae; in 34/41 foliate papilla sections and 23/24 vallate papilla sections from EGS horses, and in 38/40 foliate papilla sections and 19/20 vallate papilla sections from controls (Fig 3). In total 497 neurons were identified in EGS sections, 279 in foliate papilla sections and 218 in vallate papilla sections. In total 876 neurons were identified in control sections, 563 in foliate papilla sections and 313 in vallate papilla sections. Mean numbers of perikarya identified on each section were higher in vallate ( $\bar{x}$ =11.0; 95% C.I. 8.99-13.46) than foliate ( $\bar{x}$ =8.77; 7.22-10.65,  $P<0.001$ ) papillae. Overall, more perikarya were identified ( $P<0.001$ ) in EGS ( $\bar{x}$ =14.14; 11.63-17.18) than control ( $\bar{x}$ =7.53; 6.28-9.04) sections, however these results were associated with sample type ( $P<0.001$ ), with no difference between EGS ( $\bar{x}$ =14.77; 11.32-19.26) and control ( $\bar{x}$ =10.69; 8.48-13.47) sections if only vallate papillae was considered ( $P = 0.072$ ), but differences remaining in foliate papillae (EGS  $\bar{x}$ =13.83; 10.68-17.92, control  $\bar{x}$ =5.29; 4.03-6.94,  $P<0.001$ ).

Neurons undergoing central chromatolysis, with neuronal swelling or shrinking, cytoplasmic eosinophilia with loss of Nissl substance conferring a 'ground glass' appearance, and an eccentric, pyknotic nucleus (Fig 3a-d), without evidence of neuronophagia, glial scarring or associated inflammation, were identified in all 57 sections from 13 EGS horses which had identifiable neurons, but were absent from 56/57 control sections which had identifiable neurons. One of 6 sections of foliate papillae from a control horse with laminitis contained both normal and chromatolytic neurons, while neurons in other sections of foliate ( $n = 5$ ) and

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vallate (n = 2) papillae from this horse had normal morphology. When all sections from this horse were reviewed together blindly, the horse was categorised as a control. Examination of all H&E stained sections from each horse for chromatolysis facilitated accurate differentiation of EGS and control horses, with a sensitivity of 100% (95% CI 93.7-100) and specificity of 98.2% (90.6-100) for diagnosing EGS. Evaluation of the 2 simulated biopsies indicated that subgemmal plexi were successfully collected, with the control and EGS samples having, respectively, normal and chromatolytic neurons. Chromatolytic neurons were not immunopositive for 3'-OH ends of double- or single-stranded DNA.

Taste buds were present in the epithelium lining papillary clefts in all samples, on both walls of foliate papillary clefts and predominantly on inner walls of vallate papillary clefts (Figs 2, 4). Mean numbers of taste buds per papilla were not statistically significantly different in vallate ( $\bar{x}=6.79;5.46-8.44$ ) and foliate ( $\bar{x}=7.82;6.41-9.53$ ,  $P = 0.142$ ) papillae. Overall there were no significant differences ( $P = 0.058$ ) in taste bud numbers per papilla between EGS ( $\bar{x}=6.28;4.96-7.96$ ) and control ( $\bar{x}=8.77;6.80-11.32$ ) horses, and this lack of difference was not associated with taste bud location ( $P = 0.518$ ), with no difference between EGS ( $\bar{x}=5.64;4.32-7.38$ ) and control ( $\bar{x}=8.31;6.16-11.21$ ) horses if only vallate papillae were considered ( $P = 0.055$ ), and if only foliate papillae were considered (EGS  $\bar{x}=6.85;4.95-9.48$ , control  $\bar{x}=8.43;5.90-12.05$ ,  $P = 0.395$ ).

There was no apparent inter-group difference in taste bud morphology (Fig 4). Fine neurofilament and PGP9.5 immunopositive intragemmal fibres were present within many taste buds in vallate and foliate papillae (Fig 4b). Oval PGP 9.5-immunoreactive cells were observed within taste buds (Fig 4b), as occurs in rats [20], but contrasting with humans where PGP9.5 immunopositivity was strictly localised to nerve fibres [17]. Neurofilament

immunopositivity within taste buds was strictly localised in nerve fibres (Fig 4c). Whilst a greater proportion of taste buds which contained intragemmal neurofilament immunopositive fibres were observed in vallate (mean % = 90.9%;82.1-95.6) than foliate (80.9%;67.9-89.5,  $P < 0.001$ ) papillae, there was no significant difference between EGS (87.2%;72.8-94.6) and controls (86.1%;70.2-94.2,  $P = 0.887$ ). In addition, no significant differences between EGS and controls were observed if foliate or vallate were considered separately (foliate EGS 86.0%;61.7-96.4, control 83.6%;55.1-95.5; vallate EGS 92.6% 69.4-98.6, control 91.0%;62.4-98.4,  $P > 0.797$ ). There was no inter-group difference in % of taste buds which had apoptotic nuclei in either vallate (EGS 15.0%;7.3-28.4, control 8.1%;2.8-21.4;  $P = 0.339$ ) or foliate (EGS 5.0%;1.3-16.8, control 13.0%;5.2-29.0,  $P = 0.236$ ) papillae (Fig 4d).

There was no apparent alteration in morphology of lingual glands in EGS (Fig 5a) but % of lingual serous gland cells undergoing apoptosis was significantly increased (EGS 3.9%;1.2-12.8, control 0.1%;0.02-0.5,  $P < 0.001$ ) (Fig 5b). Apoptotic lingual mucous gland cells were not identified in sections from EGS or control horses.

## Discussion

This is the first description of the complex subgemmal neuronal network underlying equine vallate and foliate papillae. This network resembles the lingual enteric nervous system of other species [7,8].

In all EGS horses studied, many subgemmal plexus neurons had central chromatolysis, nuclear eccentricity and pyknosis, similar to that reported for enteric, autonomic, and lower motor neurons in horses with EGS [2-5]. Affected neurons were not immunopositive for the 3'-OH ends of double- or single-stranded DNA which are associated with apoptosis, suggesting that the neurodegenerative process involves necrosis rather than apoptosis.

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Blinded examination of all sections from each horse for chromatolytic neurons correctly differentiated EGS and control horses. However, the presence of a mixture of chromatolytic and normal neurons in 1/57 sections from control horses indicates that chromatolysis of subgemmal neurons is not pathognomonic for EGS, and that multiple sections per horse must be assessed to achieve diagnostic accuracy. The significance of occasional chromatolytic neurons in one section from a control horse, which was euthanased for laminitis, is unknown, however chromatolysis of central neurons has been reported in a laminitic horse [21].

Further study is required to determine the utility of histologic assessment of gustatory papillae biopsies in the pre-mortem diagnosis of EGS. Assessment of 2 post mortem 'simulated biopsies' indicated that it is possible to collect diagnostically useful samples of foliate subgemmal plexi using a uterine biopsy forceps and oral gag, and that such biopsies can facilitate differentiation of EGS and control horses. In addition, the authors successfully collected foliate papillae biopsies for clinical diagnostic purposes from a 7 year old Irish Draught gelding with a 12 day duration of clinical signs considered suggestive, but not definitive, of chronic EGS. Four biopsies were collected without apparent adverse effects using uterine biopsy forceps, under routine standing sedation, with topical anaesthesia and placement of an oral gag. Examination of 18 H&E stained sections of the biopsies, including serial sections of one biopsy, identified no neurons despite the biopsy sampling a sufficient depth of tissue to facilitate assessment of subgemmal neurons. Absence of subgemmal neurons in multiple sections was considered most likely to indicate neuronal loss attributable to EGS. Consistent with this possibility, when the horse was euthanased 24 h later, post mortem examination revealed marked paucity of neurons in the ileum. Neurons were absent from most ileal submucosal and myenteric plexi, indeed only one or 2 neurons were identified within some 3.5 cm long full thickness ileal sections. In contrast, approximately

80% of cranial cervical ganglion neurons were chromatolytic, consistent with a diagnosis of EGS. The paucity of subgemmal neurons in this horse, but not in the other AGS/SAGS cases, may have reflected the chronicity of disease, since this case was sampled later (12 days after onset of clinical signs) than the other EGS cases (median 24 h, range 6 h-5 days). This single case demonstrates that it is possible to obtain suitable biopsies from standing sedated horses. However, as with small surgical ileal biopsies [22], the difficulty determining whether absence of neurons in a biopsy reflects normal regional variation in neuronal density or neuronal loss due to EGS represents a diagnostic limitation.

While numbers of subgemmal neurons differed significantly between EGS versus control horses and between foliate versus vallate papillae, no conclusions can be made regarding relative densities of neurons because the areas counted were not standardised.

Sections from horses with AGS/SAGS, and not chronic EGS, were evaluated in the study because it was considered that the early, severe cases were most likely to have evidence of neuronal chromatolysis, and since the authors consider that the diagnosis of AGS/SAGS is more challenging than that of chronic EGS. Further study is required to evaluate the subgemmal neuronal plexus in chronic EGS.

This is the first report of chromatolysis of subgemmal neurons in a spontaneous neurodegenerative disease. Several human neurological diseases are associated with reduced numbers of lingual gustatory papillae (Machado-Joseph disease, Stüve-Wiedemann syndrome, familial dysautonomia, dystonia musculorum, Behçet's disease) and/or diminished taste (diabetes mellitus, Alzheimer's disease, Huntington's disease, hereditary sensory and autonomic neuropathy type IV) [23]. This is likely a consequence of defects in neurotrophic

support from innervating neurons which is essential for development and survival of gustatory papillae [23,24]. While the numbers of intragemmal fibres within taste buds are reduced in Alzheimer's disease [17], there are no reports of subgemmal plexus neurodegeneration in these diseases. The recognition of subgemmal neuronal chromatolysis in EGS likely partly reflects the acute severe neural insult which characterises EGS, contrasting with the relatively slower neurodegeneration occurring in the aforementioned human diseases.

In other species, subgemmal plexus neurons regulate lingual glands [8,10], unique minor salivary glands that secrete a variety of proteins with roles in taste transduction, digestion and antimicrobial protection of lingual epithelium [25-28]. EGS horses had no apparent alteration in lingual mucous and serous gland morphology, but had increased proportions of lingual serous gland cells undergoing apoptosis, the first time this pathology has been reported in a spontaneous neurodegenerative disease. In other species lingual gland receive parasympathetic innervation via pre-ganglionic neurons originating in the inferior salivatory nucleus and travelling within CN IX, with post-ganglionic neurons originating in the subgemmal plexus, while sympathetic innervation is via post-ganglionic neurons originating in the cranial cervical ganglion and travelling within CN XII [25,28,29]. Serous gland apoptosis in EGS is potentially a consequence of loss of neurotrophic support, since there is neurodegeneration of post-ganglionic sympathetic neurons originating within the CCG and post-ganglionic parasympathetic neurons within the subgemmal plexus. Consistent with this possibility, experimental transection of CN XII induced ultrastructural degeneration of lingual glands without altering their light microscopic appearance [28]. Since lingual glands likely modulate responses of taste receptors by altering the micro-environment in which taste transduction occurs [7,8,25], it is possible that subgemmal plexus neuronal chromatolysis and

associated alterations in serous lingual glands in EGS could attenuate chemosensory capacity in EGS and potentially contribute in part to the dramatic reduction in food intake which accompanies EGS.

Despite the development and turn-over of taste buds and lingual papillae being extremely sensitive to the function of their innervation [23,24], EGS was not associated with detectable alterations in taste bud numbers or morphology, proportion of taste buds containing intragemmal neurofilament immunopositive fibres, or proportion of taste buds containing apoptotic cells. Furthermore EGS did not induce the alterations of taste bud morphology which characterise apoptosis following experimental CN IX neurectomy [18], suggesting adequacy of CN IX-mediated neurotrophic support to taste buds and papillae at the time of sampling.

While identification of chromatolytic subgemmal neurons in post mortem samples correctly differentiated all EGS and control horses in this study, further study is required to evaluate this technique for pre-mortem EGS diagnosis.

#### **Authors' declaration of interests**

No competing interests have been declared.

#### **Ethical animal research**

Tissue was collected with horse owners' consent at necropsy from horses undergoing humane destruction. The School Ethics Committee approved the study.

### Source of funding

This project was funded by the Equine Grass Sickness Fund ([www.equinegrasssickness.org.uk](http://www.equinegrasssickness.org.uk)).

### Acknowledgements

We thank Sharon Moss for immunohistochemistry and Dr Caroline Hahn for reviewing the manuscript.

### Authorship

All authors qualify for authorship on the grounds of the following: (i) study design [B. McGorum, R.S. Pirie, A. Cox], (ii) study execution [all authors], (iii) data analysis and interpretation [B. McGorum, R.S. Pirie, D. Shaw, A. Cox], (iv) preparation of the manuscript [B. McGorum, D. Shaw, A. Cox], and (v) final approval of the manuscript [all authors].

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<sup>a</sup>AbD Serotec, Kidlington, UK.

<sup>b</sup>Dako, Ely, UK.

<sup>c</sup>Millipore, Watford, UK.

<sup>d</sup>The R Foundation for Statistical Computing (<http://www.r-project.org/foundation/>).

### Figure legends

**Fig 1:** Dorsal view of excised tongue showing vallate and foliate papillae.

**Fig 2:** Low power view of (a) vallate papilla from a control horse, and (b) foliate papillae



from an EGS horse. There is a complex subgemmal plexus of PGP 9.5 immunopositive neurons (arrow head) and nerve fibres. Superficial (Sup) and deep (Deep) ganglia, lingual serous glands (SG) and mucous glands (MG), and muscle (M) are evident. Taste buds located on the gustatory epithelium in the papillary clefts (PC) are innervated by a particularly dense network of PGP 9.5 immunopositive fibres (arrows). PGP 9.5 immunohistochemistry. Bar = 1mm.

**Fig 3:** Superficial (a and b) and deep (c and d) subgemmal neurons from control (a and c) and EGS (b and d) horses. Neurons from EGS horses are chromatolytic. H&E. Bar = 100µM.

**Fig 4:** Gustatory epithelium of foliate papilla, showing (a) taste buds lining the papillary cleft [arrows]. Control horse. H&E, (b) fine PGP9.5 immunopositive intragemmal fibres and oval PGP 9.5-immunopositive cells [arrow] within taste buds. EGS horse. PGP9.5 IHC, (c) fine neurofilament immunopositive intragemmal fibres [arrow] within taste buds. EGS horse. Neurofilament IHC, and (d) Apoptotic cells within taste bud [long arrow] and squamous epithelium [short arrow]. ApopTag Peroxidase In Situ Apoptosis Detection method. Control horse. A-c bar = 100µM.

**Fig 5:** (a) Lingual serous gland underlying the vallate papillae from a control horse (H&E). (b) Apoptotic cells (arrows) within lingual serous glands underlying the vallate papilla of an EGS horse. ApopTag Peroxidase In Situ Apoptosis Detection method.

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